PROTEIN QUALITY CONTROL AT THE ENDOSOME:

A NOVEL MODEL

by

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STATEMENT OF THESIS APPROVAL

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ABSTRACT

Transporters must be sustained in proper working function at the plasma membrane to prevent serious harm to a cell. A dysfunctional transporter may cause leaking of ions, loss of membrane potential, disturbed cell signaling, and even cell death. Therefore, cautious monitoring of transporters occurs at the plasma membrane. A transporter which becomes unfolded or unstable is quickly endocytosed and taken to the early endosome compartment of the cell. However, these unfolded transporters are not automatically degraded, as a cell attempts to preserve them by allowing time for refolding. It is this process we refer to as quality control: where the decision is made whether to degrade the dysfunctional protein to maintain cell integrity or attempt to refold it to conserve energy. The endosome provides a safe place where this decision can be made. Properly folded and functional transporters will return to the plasma membrane to resume pumping, while those deemed dysfunctional will continue on to be degraded.

In the case of the yeast uracil transporter Fur4, unfolded/destabilized Fur4 is ubiquitinated (signal for degradation added) by Rsp5, the only ubiquitin ligase known to work at the plasma membrane in yeast. After being endocytosed and brought to the early endosome, the decision to degrade or recycle Fur4 is made there. How exactly that quality control decision is made is not well understood.

It is known that ubiquitination status plays a major role in that decision, and that both ubiquitination and deubiquitination can occur at the early endosome. However, studies have shown that Fur4 can recycle in spite of being in a ubiquitin tagged state in some mutant strains, suggesting the quality control decision is not so black and white.

We attempt to elucidate this quality control process. Our results, and those published, have led us to a model whereby ubiquitination status is not the sole deciding factor involved in sorting at the early endosome, but where retention of ubiquitinated cargo is actually the key sorting step. Herein we describe a complex of proteins working together at the early endosome to carry out quality control.

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ABBREVIATIONS

5-FU 5-FluoroUracil

CFTR ..... Cystic Fibrosis Transmembrane conductance Regulator

DUB ..... DeUBiquitinating enzyme

ER ..... Endoplasmic Reticulum

ESCRT ..... Endosomal Sorting Complex Required for Transport

EQRS ..... Endosomal Quality control & Retention System

GFP ..... Green Fluorescent Protein

ILV ..... IntraLumenal Vesicle

LID ..... Loop Interaction Domain

MCS ..... Multiple Cloning Site

MVB ..... MultiVesicular Body

PM ..... Plasma Membrane

QC ..... Quality Control

RFP ..... Red Fluorescent Protein

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CHAPTER 1

INTRODUCTION

1.1 Protein Homeostasis

Protein homeostasis, or proteostasis, is the maintenance of well functioning proteins in a cell required for its success and survival. Unfolded proteins are a detriment to the cell because of loss of function, formation of protein aggregates, and triggering of cellular stress responses. Unfolded proteins at the plasma membrane are especially detrimental to a cell, as they can cause leaking of ions and loss of membrane potential across the plasma membrane (PM). Therefore, a cell must remove unfolded proteins from the plasma membrane immediately through what is called endocytosis, and eliminate the dysfunctional proteins through degradation. The downside to this overly cautious system is that it can be energetically costly. Degraded proteins have to be replaced through new protein synthesis (which requires a lot of energy) to maintain functioning proteins at the plasma membrane. To conserve energy, proteins that are endocytosed are not always degraded. These proteins are given time to refold, safely inside the cell, and then can be recycled back to the plasma membrane. This process allows for the balance of cell integrity with energy conservation. A cell can remove potentially harmful proteins from the

plasma membrane, reevaluate/refold them, and then recycle and reuse the functional proteins while degrading the dysfunctional (*1*). This system, whereby unfolded proteins are taken care of by being either refolded or degraded, is referred to as protein quality control (QC).

There are different, but similar, protein quality control systems in place at other compartments in the cell as well as the plasma membrane. The system in place at the Endoplasmic Reticulum (ER) acts as new proteins are synthesized and folded, before they are transported to their destination in the cell. Generally, unfolded/misfolded proteins at the ER are held back and reevaluated by chaperone proteins, which assist in refolding, or when unsuccessful, aid in the protein being sent for degradation (*2*, *3*). The idea is similar to that at the plasma membrane, but the proteins involved in quality control, and the processes by which they work, are very different at each location. For one thing, chaperones may not have as much access to membrane proteins at the endosome, so other QC mechanisms may be in place. While ER quality control still lends itself to many unanswered questions, also obscure is the quality control system that works after a protein has reached the plasma membrane.

The importance of having a protein quality control system in place is evident in diseases that result from loss of proteostasis. Alzheimer's disease, Parkinson's disease, cystic fibrosis, prion diseases, and many more, all involve protein un-/mis-folding or protein aggregation (*4-6*). Whether the observed imbalance in proteostasis is a causative factor or symptomatic attribute of the disease is often unknown. Cystic fibrosis, however, is a clear example of the direct effects of protein misfolding. A ΔF508 mutation, which renders the

mammalian cystic fibrosis transmembrane conductance regulator (CFTR) protein unable to fold properly, is present in the vast majority of cystic fibrosis patients (*7*). Even native CFTR, a large and cumbersome protein, simply takes too long to fold efficiently, and so it is often subjected to ER quality control and never makes it out of the ER. Accordingly, about 75% of $CFTR^{\Delta F508}$ protein is unable to fold and is held back in the endoplasmic reticulum (*8*). Hence, not enough of the protein is able to make it to the plasma membrane where it serves as a chloride channel, and the repercussion is an imbalance of chloride ions, leading to many problems.

The native CFTR protein that escapes the ER and makes it to the plasma membrane is still very unstable and frequently endocytosed (*9*). CFTR has large cytosolic domains, and less region within the membrane, so it is able to be accessed by chaperones post-Golgi. About 75% of the CFTR endocytosed is, in turn, recycled back to the plasma membrane (*10*). So, the route of recycling instead of being degraded is very common for some proteins, and may be the norm. It is also a vital pathway, as the degradation of all endocytosed native CFTR would also likely result in a cystic fibrosis phenotype, one would predict. Clearly, there is a very important quality control process going on directly after endocytosis, about which we know very little, and it will be discussed herein.

1.2 Trafficking of Plasma Membrane Proteins

Proteins have predefined destinations in a cell, and specific routes to travel in getting there. Proteins are tagged with specific amino acid sequences that specify where they should be sent (called sorting signals) (*11*), much like an

address on a postal package. Generally, ER targeted membrane proteins that lack specific sorting signals are sent to the plasma membrane as a default pathway(*12*). Other membrane proteins contain signals to send them to specific compartments in a cell. This sorting decision occurs at the “post office” of the cell, the Golgi apparatus.(*12*)

After being synthesized in the ER, membrane proteins are sent through the Golgi for packaging and, if lacking a sorting signal, then travel in vesicles out to fuse with the plasma membrane (*12*). If the protein becomes unfolded or unstable at the PM, it is ubiquitinated (signal for degradation added), endocytosed, and taken to the early endosome (Fig. 1.1a) (*13-16*). The early endosome likely serves as a safety compartment in the cell, as it is here that a protein can be reevaluated, and a decision made whether to recycle or degrade the protein (*14*, *17*). If the protein appears folded, it can be deubiquitinated (signal for degradation removed), and recycled from the early endosome back to the PM, to continue functioning there (Fig. 1.1b) (*17*). If the protein is unable to refold, it continues down the pathway to degradation (Fig. 1.1c). The early endosome matures into a late endosome, while taking its cargo (contents) with it (*15*). At the late endosome stage, the Endosomal Sorting Complex Required for Transport (ESCRT) proteins are recruited (*18*). The ESCRTs concentrate cargo proteins into a pool on the endosomal membrane (*15*, *19*). The ESCRTs also facilitate the deformation of the membrane, to create an invagination into which the deubiquitinated cargo is deposited (Fig. 1.1d). For cargo to be deposited into intralumenal vesicles (ILVs), ubiquitin, the modification that has been added onto the cargo protein as a degradation signal, is removed and recycled (*20*, *21*). At

this stage, in which formation of intralumenal vesicles (ILVs) occurs, the late endosome has matured into what is called a multivesicular body (MVB) (*19*). The MVB then fuses with the lysosome (in higher eukaryotes, vacuole in plants/fungi), and releases its ILVs there (Fig. 1.1e) (*1*, *19*). In the acidic lumen of the lysosome/vacuole, ILVs are exposed to lipases and peptidases, which degrade both the lipids and the proteins, respectively (*1*, *19*). From there, the broken up amino acids can be recycled and go to the cytoplasm for new protein synthesis.

Ubiquitin, as previously mentioned, is considered the universal tag for degradation (*22*). The 76 amino acid ubiquitin moiety is added to both soluble and membrane proteins as a signal to the cell that they need to be degraded (*17*, *23*). A single ubiquitin is sufficient as the signal for degradation of membrane proteins (*24*), but polyubiquitin chains are common (*15*, *25*). Three types of enzymes act to add the ubiquitin modification to target proteins: E1, E2, and E3 enzymes. E1 enzymes are ubiquitin-activating enzymes, which prepare ubiquitin for addition to a target protein. E2 enzymes are ubiquitin-conjugating enzyme, which are responsible for chain assembly and elongation (*26*). E3 enzymes are the ubiquitin ligases – they covalently attach the ubiquitin moiety onto a target protein, specifically at a lysine residue (*27*, *28*).

In the yeast *Saccharomyces cerevisiae*, there is only one known E3 ubiquitin ligase that works on proteins at the plasma membrane, called Rsp5 (*29-32*). RSP5 knockout strains are not viable (*31*), but an *rsp5-1* strain contains a hypomorphic allele of RSP5 that results in a far less active ubiquitin ligase (*33*). In this strain, proteins are stabilized on the plasma membrane because they are unable to be efficiently ubiquitinated (*16*, *34*, *35*). Mutation of the lysine residues

within a protein that are targeted for ubiquitination also results in the stabilization of the mutant protein on the plasma membrane (*35*, *36*).

Either monoubiquitin or polyubiquitin chains may be formed on a cargo protein. Polyubiquitin chains can be made through differing linkages as well, meaning another ubiquitin can be added onto any one of the 7 lysines within ubiquitin (*37*). All of these variations may signal different fates for target proteins (*38*). The standard convention is that K63 polyubiquitin chains are typically formed for the degradation of transmembrane proteins through the MVB pathway, while K48 linkages are used to direct soluble proteins to the proteasome for degradation (*15*, *39*, *40*).

1.3 Fur4 as a Model Protein in Yeast

To study quality control at the plasma membrane, we work with the yeast *Saccharomyces cerevisiae* as our model organism. *S. cerevisiae* is a good choice as it is hardy, inexpensive, and easy to genetically modify as we keep it in the haploid state. Our chosen model protein is Fur4. Fur4 is the yeast uracil transporter. It is a member of the nucleobase cation symport-1 (NCS-1) family; it pumps uracil in along with a proton (symport), using the proton gradient to move uracil against its gradient (*41*, *42*). Fur4, at 633 amino acids, is a large transporter, containing 12 transmembrane domains (*43*). Because the transport of uracil requires large conformational changes in Fur4, this protein is rather unstable. The physical arrangement of these domains and loop regions, and their movements during pumping, can be estimated based on knowledge of the crystal structure of a NCS-1 family bacterial transporter homolog, Mhp1 (*42*, *44*).

Based on that structure, Keener & Babst (2013) predicted Fur4 to have an N-terminal LID region, or Loop Interaction Domain, which as it sounds, interacts with the loop regions of the transmembrane domains on the cytosolic side (*35*). Also present on the N-terminus is a degron, a series of phosphorylation and ubiquitination sites (lysines); presence of which is sufficient for recognition and degradation of a target protein (*35*, *45*, *46*) (Fig. 1.2).

Many studies have found that the yeast uracil permease Fur4 is downregulated upon addition of substrate (i.e., uracil) to the culture (*16*, *35*, *47*, *48*). Downregulation efficiency was similar to that seen with heat shock or hydrogen peroxide treatment, known triggers of protein unfolding and endocytosis (*35*). Keener & Babst (2013) proposed that the flexible Fur4 must go through large conformational changes to allow the transport of uracil, and during this time out of the ground state, the protein may look unfolded (*35*). Their idea is that the LID region actually acts like a lid on Fur4, keeping closed in the ground state (*35*). In the ground state, within the N-terminal 110 amino acids are residues that make contact with the loops of the transmembrane domains; this interaction is what holds the LID "shut" while also contributing to the protein's stability (*35*). While the LID is "shut" the degron is tucked in, preventing its accessibility (*35*) (Fig. 1.2a). But during pumping of substrate, the LID must open up to allow binding and release of uracil to the cytosol. During this time spent open, the LID-loop interactions are lost and the degron is exposed, and thus accessible to ubiquitination (*35*) (Fig. 1.2b). Under this model, simple diffusion and chance determine ubiquitination probability. During degron exposure, if the ubiquitin ligase Rsp5 comes along, it will ubiquitinate Fur4. Therefore, longer

time spent in the non-ground state equals longer degron exposure, and greater chance of a run in with Rsp5, which leads to ubiquitination and endocytosis. This is called an intrinsic sensing mechanism in that Fur4 itself senses when it is unfolded by loss of LID-loop interactions and degron exposure, and thus presents iteslf for ubiquitination (*35*). This QC mechanism works independently of chaperones, as opposed to CFTR quality control (*1*, *35*).

Support for the LID-degron idea comes from experimental manipulation of Fur4 protein. Deletion of the N-terminal 60 amino acids, what is deemed the degron, resulted in stabilization of Fur4 on the plasma membrane, even under conditions that should result in its downregulation (e.g., heat shock, peroxide treatment, and high substrate addition) (*35*). Alternatively, lysine-to-arginine mutation of residues within the degron also results in Fur4 stabilization under these conditions (*35*). This demonstrates that the degron, and specifically lysine ubiquitination sites, are necessary for Fur4 downregulation. Fur4 in the *rsp5-1* strain is also stabilized at the plasma membrane, in spite of cell stress and substrate addition (*35*). This shows ubiquitination is necessary for Fur4 downregulation.

Since we have a supported model of Fur4 behavior at the plasma membrane, it is a good transporter to use in our studies. Additionally, its flexible nature and instability make Fur4 a model protein for our quality control experiments: unfolding can easily be induced, causing ubiquitination and endocytosis of Fur4.

Endocytosis of Fur4 can be induced by three modes: 1) cell starvation, which is known to cause downregulation of nutrient transporters; 2) stressing the

cell via heat shock, peroxide treatment, etc.; or 3) addition of high uracil concentration. The first mode is understood to occur as part of starvation response pathways like TORC1 (Target Of Rapamycin Complex 1), which are involved in nutrient sensing and turnover of proteins in response to low nutrients (*49*). The second mode is more straightforward – the use of known cell stressors to cause protein unfolding by disrupting chemical interactions that hold the protein in its folded conformation. The third mode, substrate induced downregulation, had been observed previously, but was not well understood until the new mechanism was proposed and published by Kenner & Babst in 2013 (*35*). The second and third modes both use the same mechanism resulting in endocytosis – the LID-degron system described above – and thus, these are the modes we employ to induce endocytosis in QC experiments (*35*).

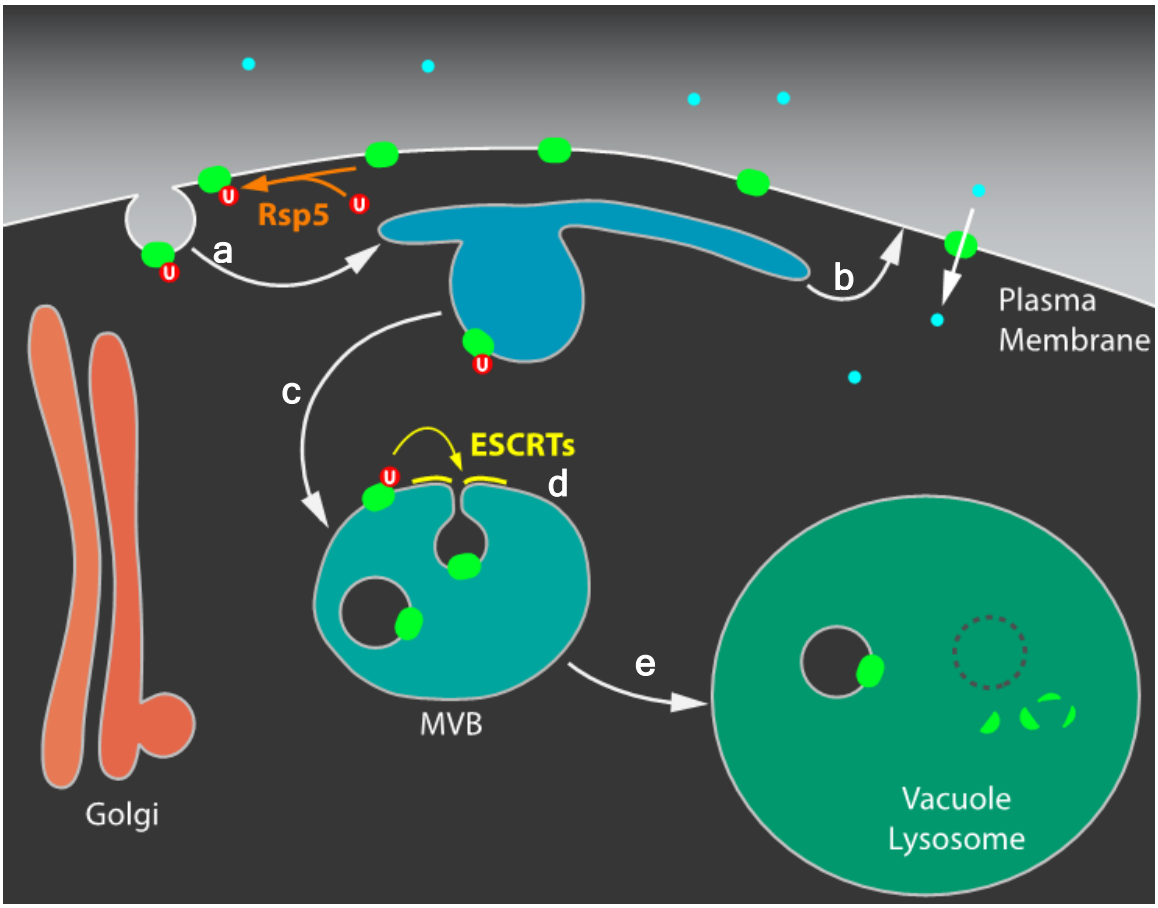


Fig. 1.1: General Trafficking Route of a Plasma Membrane Protein
a. A plasma membrane protein undergoes endocytosis, and is sent to the endosome. b. Endosomal membrane recycles, and can carry cargo protein with it, to fuse back with the plasma membrane. c. Alternatively, a protein continues down the path to degradation, traveling from endosome to MVB. d. ESCRTs are recruited to facilitate deposition of cargo proteins into ILVs. e. MVB fuses with vacuole/lysosome, releasing its contents for degradation there.
(Figure provided by Markus Babst.)

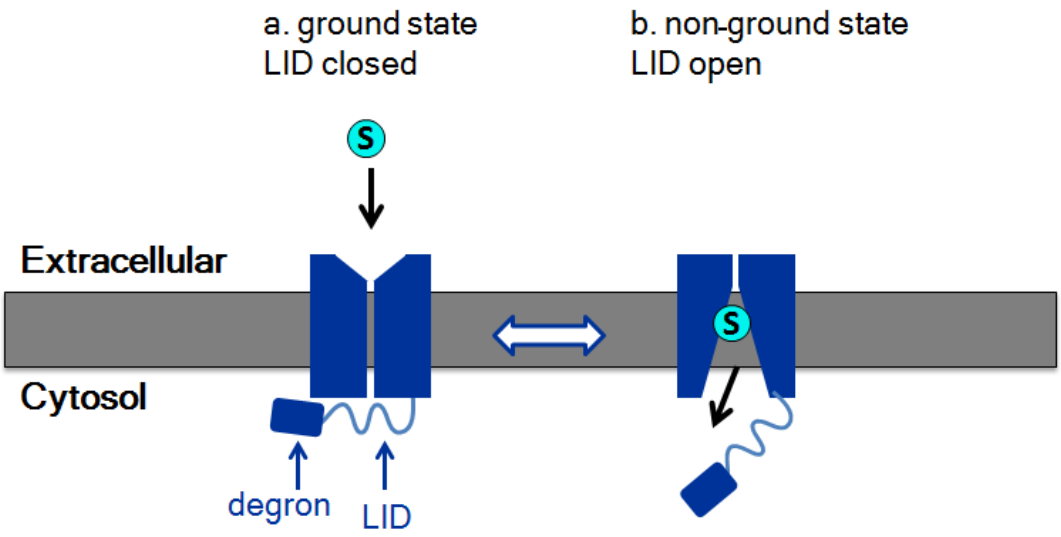


Fig. 1.2: Fur4 Conformational Changes
Without substrate bound, Fur4 is in the ground state, with LID closed and degron tucked away from Rsp5 access. During substrate pumping, Fur4 undergoes large conformational changes that cause the LID to open up, exposing the degron. Fur4 deviates from the ground state any time uracil binds, but can revert to the ground state when no longer bound.

CHAPTER 2

A NOVEL MODEL: ENDOSOMAL QUALITY CONTROL AND RETENTION SYSTEM

2.1 Introduction

2.1.1 Early Endosome Dynamics

After endocytosis of a plasma membrane protein, it travels to the early endosome, where a decision is made whether to continue down the path to degradation or to recycle back to the plasma membrane. But how is this QC decision made and carried out? In the case of Fur4, we understand the quality control mechanism in place at the plasma membrane, but we do not know much about the second QC system in place at the early endosome. There is plenty of evidence of dynamic ubiquitination and deubiquitination of cargo proteins, which points to a method of executing this decision (*1*, *25*, *50*, *51*). At the early endosome, ubiquitin ligase can add ubiquitin moieties to a protein, while ubiquitin proteases can cleave off ubiquitin moieties. Again, the only known ubiquitin ligase working at the early endosome is Rsp5 (*29-32*). There are many deubiquitinating enzymes (or DUBs) in a cell – potentially around 100 in human and 17 in yeast – possibly of gene families that evolved to function at different compartments of a cell or on specific substrate (*52*, *53*). We focused on

deubiquitinating enzymes Ubp2 and Ubp7 as likely to be working at the early endosome, based on evidence of their action on Ede1, an endocytic protein, and based on their interactions with proteins known to be functioning around this stage (*25*, *54*).

A simplistic idea follows that upon reaching the early endosome, a cargo protein either is deubiquitinated and recycled back to the plasma membrane, or it is reubiquitinated/polyubiquitinated and goes along with the maturing endosome to be degraded in the vacuole. In this scenario, ubiquitination status is the key sorting signal to determine protein fate. Degradation is the signal mediated path, requiring presence of ubiquitin (the signal), while recycling to the plasma membrane is the default path taken in absence of ubiquitin. If this is the case, the system could be upset by perturbation of the enzymes Rsp5, Ubp2, or Ubp7. Removal of Rsp5 from the system would, we expect, result in cargo proteins lacking ubiquitin sorting signals, and recycling by default back to the plasma membrane. (This idea of course is convoluted by the fact that Rsp5 works at the plasma membrane too, so initial endocytosis would be largely impaired.) On the other hand, removal of the DUBs from the system would result in cargo proteins remaining ubiquitinated or growing long polyubiquitin chains, and going forth with the maturing endosome to be eventually degraded in the vacuole. This is what is expected if quality control at the early endosome is a straightforward system whereby ubiquitin ligase adds ubiquitin to a protein that appears unfolded, and DUBs remove ubiquitin from an apparently folded protein. In the case of Fur4, and based on the work done by Keener & Babst (2013), “appearing unfolded” means LID opening and degron exposure, while appearing folded means the

degron is neatly tucked away, inaccessible to Rsp5. So, the decision made at the early endosome would be dependent on whether a protein appears unfolded or folded and on corresponding ubiquitination status.

Rechecking of proteins at the early endosome is very important as this step allows the balance of cell integrity with energy conservation, and it seems to be the stage when the final decision is made on a protein's fate. It is also a very common process to recycle PM proteins, as in the example of CFTR that was given (*10*, *55*). Proteins may become temporarily unfolded, but they can be safely brought inside the cell for reevaluation by the second QC system, and then ubiquitin ligases/proteases can modify the protein accordingly to facilitate the decision. However, the quality control scenario presented is a very simplistic idea and current research suggests the process is not so black and white.

2.1.2 Key Proteins Involved at the Early Endosome

To delve into the complexity of the quality control system at the early endosome, a deeper look at the proteins at work is warranted. There is plenty of evidence that Rsp5, Ubp2, and Ubp7 do not work independently of each other or of other proteins (*25*, *50*, *51*, *56*). On the contrary, many of these proteins physically interact with each other (*25*). Pull down assays (with addition of mutation of predicted binding sites) plus immunoblotting were used to develop a protein interaction map, as published (*25*). That data, in addition to similar experiments, informed the interaction map shown in Fig. 2.1. According to the map, Rsp5 and Hua1, a protein of unknown function, physically interact with each other and with Hse1, an ESCRT-0 protein (*25*). Both proteins also interact with

Sla1, part of the upstream endocytic machinery (*57*). Ubp7 interacts with Hse1 as well (*25*). The protein Rup1 stands for Rsp5-Ubp2 interaction Protein; essentially, Rup1 tethers the ubiquitin ligase to the DUB (*50*). The importance of these interactions has not been fully elucidated yet, but it is known that their interaction is important for controlling sorting efficiency. For example, it seems Rsp5 requires Rup1 for tethering to Ubp2, in order for Rsp5 to efficiently ubiquitinate cargo (*50*, *51*). It has been proposed that this may be due to an autoubiquitination process carried out by Rsp5 (*56*). The idea is that through autoubiquitination, Rsp5 could actually downregulate itself, adding the signal for degradation to itself, and then Ubp2 acts on Rsp5 by removing the ubiquitin it adds to itself. But others argue this is not the reason for Rsp5's requirement of Ubp2 for efficient ubiquitination of cargo, due to the fact that deletion or overexpression of Ubp2 does not alter steady state levels of Rsp5 (*50*).

The four main proteins we have focused on are Rsp5, Rup1, Ubp2, and Hua1. All are soluble proteins and all are recruited to the endosomal membrane (according to a mass of data from the *Saccharomyces* Genome Database). We think these four proteins function in quality control at the endosome. We believe they work after the upstream endocytic machinery, and before the downstream ESCRT machinery takes over. But how exactly they function together at the endosome is unknown.

2.1.3 New Model of QC at the Endosome

How do the ubiquitin ligase and DUBs work together at the early endosome to determine protein fate? This is the big question that the present

study attempts to address. The simple model of dynamic ubiquitination and deubiquitination does not suffice. Evidence at odds with this simple model is abundant. The biggest problem is that UBP2 deletion experiments have shown increased recycling of cargo, and less degradation (*51*). Published growth assays have shown hypersensitivity to drugs in *ubp2*Δ cells, indicating a greater presence of the transporter at the plasma membrane, indicative of increased recycling (*51*). Pulse chase experiments confirmed this, with Fur4-GFP remaining on the plasma membrane even after addition of uracil substrate (*51*). This is in stark contrast to what is expected. Deletion of the deubiquitinating enzyme Ubp2 should lead to more ubiquitination and thus more degradation of plasma membrane proteins, but only the first prediction holds true. Deletion of UBP2 does result in increased polyubiquitination, as demonstrated through immunoblotting against ubiquitin in *ubp2*Δ cells (*58*). But in looking at Fur4, it did not lead to concomitant increased degradation as expected. There is clearly more going on at the early endosome than just ubiquitination versus deubiquitination of cargo proteins. The goal of this study was to attempt to reconcile these unexpected results and explore new models of quality control at the early endosome.

Based on current knowledge, we came up with a new model whereby the aforementioned proteins – particularly Rsp5, Ubp2, Hua1, and Rup1 – work in a complex to retain cargo proteins at the early endosome. We have dubbed this model EQRS = Endosomal Quality control & Retention System. The idea is that while a cargo protein goes through a cycle of ubiquitination and deubiquitination, it is transiently bound to Rsp5 or Ubp2, and likely bound to Rup1 or Hua1 through

attached ubiquitin moieties. All of these interactions keep the cargo protein at the early endosome. After a period of cycling, whatever remains will continue with the maturing endosome, through the MVB pathway, to eventually be degraded. While ubiquitin is still the signal for degradation, and lack thereof should default to recycling, under this model, retention is the key sorting step. So disruption of EQRS components could disrupt the whole complex and thus retention, causing a loss of regulated sorting at the endosome. (Fig. 2.2) The model draws comparisons from the system of glycosylation at the ER, whereby keeping a soluble protein in a cycle of glycosylation and de-glycosylation reactions retains the protein in the ER (*2*).

2.2 Materials and Methods

2.2.1 Strains & Growth Conditions

Saccharomyces cerevisiae strains used for this study are listed in Table 2.1. Strains were created as follows: PCR was done on integration plasmid templates containing a selectable marker (e.g., G418 resistance, HIS auxotrophy), and fluorescent tag where needed, with primers designed to add 50 bp flanking regions of homology from target genes; transformation into wildtype yeast strain SEY 6210 allowed homologous recombination of the marker/tag into the genome; selection markers allowed for picking of colonies with the edited genome. New strains were confirmed by PCR analysis of genomic DNA. Yeast strains were grown at 30°C in common YPD (Yeast extract-Peptone-Dextrose), or in YNB (Yeast Nitrogen Base) supplemented with all amino acids except auxotrophic marker(s) to maintain plasmid. For inducing expression of copper

promoted Fur4-GFP (pJK19), 50 µM copper sulfate was added to the growth media. Induction of substrate-induced downregulation was done by adding 2g/100ml uracil to the media. Heat shock treatment involved swirling culture tubes in a 37°C water bath to shock, and then transfer to a 37°C shaking incubator for between 15-45 minutes.

2.2.2 DNA Manipulations

Plasmids used for this work are listed in Table 2.1. Genes were amplified by PCR from genomic SEY6210. Restriction enzyme cloning was then used to insert the gene into a pRS4 series vector, with promoters as specified in Table 2.1. Plasmids were amplified in XL1-Blue strain of *Escherichia coli*, grown in standard LB media at 37°C, and selected for with addition of 100g/ml ampicillin to the media. For construction of the $ubp2^{C745A}$ mutant: a plasmid was created through PCR mutagenesis and homologous recombination. PCR amplification of two fragments of UBP2 was done on a UBP2 containing plasmid (pKF17), using M13R universal primer as forward with a UBP2 reverse primer with base substitution, and T7 universal primer as reverse with a UBP2 forward primer with base substitution. Use of M13R and T7 created regions of homology to the MCS of a pRS vector. The two PCR fragments could then be transformed with a gapped vector into yeast, and homologous recombination stitched together the three fragments, creating the plasmid pKF19. pKF19 size was confirmed by plasmid prep and test digest, and sanger sequencing confirmed presence of the C745A mutation.

2.2.3 Fluorescence Microscopy

A deconvolution microscope (DeltaVision, Applied Precision) was used to perform all fluorescent microscopy in the present study. Cells were typically grown to 0.6-0.8 OD_{600}/ml (log phase) before harvesting for microscopy. The different strains were analyzed for Fur4-GFP cellular distribution, observing presence/absence and relative amount of protein in compartments along the endocytic trafficking route: plasma membrane, endosomes, MVBs, vacuole.

2.2.4 5-Fluorouracil (5-FU) Growth Assays

YNB plates were made without uracil (and without specific plasmid selection markers when required), and 5-Fluorouracil (5-FU) was added to the plates prior to pouring. 2µM 5-FU was determined to be the optimal concentration for these growth assays. Cultures were allowed to grow to ~0.7 OD_{600}/ml before harvesting for the assay. Cells were diluted in 1M sorbitol to a final concentration of 0.5 OD_{600}/1ml. A 1:5 dilution series was done in sorbitol in a 96-well plate. 3µl of the cells in sorbitol were plated, and images were captured after 1-4 days of growth at 30°C.

2.2.5 Western Blotting

Cells were grown to log phase and a total of 3 OD_{600} of cells were harvested for protein preps. After centrifiguation, cells were resuspended in 6M urea sample buffer plus 100µl/ml β-mercaptoethanol. Cells were vortexed with glass beads and heated at 65°C for 5 minutes (2x). 8% SDS-PAGE gels were used for visualizing Fur4-GFP. Primary antibodies used were mouse-αGFP and

rabbit-αSnf7 (loading control), while secondary antibodies were goat-αmouse (800 channel) and goat-αrabbit (680 channel). Blots were imaged on Licor Odyssey scanner and analyzed with Image Studios 4.0.

2.3 Results

2.3.1 Localization of EQRS Components

Our first priority was to confirm that the four key proteins in the EQRS complex are recruited to the endosome. Rsp5 has already been localized to endosomes, as well as the plasma membrane (*32*). To localize the other components, we made genomic integrations of GFP tags on RUP1, HUA1, and UBP2. Hua1-GFP had such low and diffuse expression, it was difficult to tell whether it specifically localized to the endosome. Rup1-GFP and Ubp2-GFP were also diffuse in the cell, but possibly appeared concentrated in small dots near the plasma membrane (likely endosomes). For further proof, we looked at the GFP-tagged proteins in a *vps4*Δ strain (which accumulates late endosomes), and co-localized with mCherry-DID2 (an ESCRT factor tagged with an RFP). There was no overlap of the two fluorescent markers, indicating that Rup1 and Ubp2 are not in late endosomes, but work upstream of the ESCRTs (Fig. 2.3).

2.3.2 Fur4-GFP Trafficking in Hypomorphic rsp5-1

Use of an hypomorphic RSP5 allele results in stabilization of Fur4 on the plasma membrane, even under heat shock (Fig. 2.4a). However, when we look at Fur4-GFP in *rsp5-1* strain MYY808 by microscopy, there were still some endosomal structures visible after 60 minutes +uracil (Fig. 2.4b). In this strain,

Rsp5 activity is severely impaired, but not abolished. So, Rsp5 can still ubiquitinate at the PM, though much less efficiently, and limited endocytosis does occur. However, once at the early endosome, Rsp5 always loses the tug-of-war with the DUBs, and thus proteins are deubiquitinated and recycle. The observed phenotype in *rsp5-1* strain represents both stabilization of Fur4 on the plasma membrane and increased recycling of Fur4 to the PM.

2.3.3 Deletion of EQRS Component Ubp2

Deletion of UBP2 results in increased polyubiquitination. Since ubiquitin is supposed to be the signal for degradation, it is surprising that deletion of UBP2 does not lead to increased degradation as expected. Our retention model is based on this anomaly and attempts to explain it. Therefore, while it has been published, we wanted to confirm it with our own experiments.

We looked by fluorescence microscopy at trafficking of Fur4-GFP in a UBP2 deletion strain (*ubp2*Δ, named MCY66) and observed similar effects (Fig. 2.5). Fur4 was stabilized on the plasma membrane, in spite of treatments like heat shock or substrate addition, which usually induce downregulation. In fact, the *ubp2*Δ cells looked more like *rsp5-1* cells (Fig. 2.4) than like wildtype cells (Fig. 2.5). Therefore, two strains that are expected to have polar opposite phenotypes, if Rsp5 functions solely as a ubiquitin ligase and Ubp2 functions solely as a deubiquitinase, actually yield very similar phenotypes. This indicates additional functions for these proteins at the early endosome.

Additionally, we used growth assays to appraise relative amount of transporters on the plasma membrane. Transporters like Fur4 do not have an

on/off switch; if they are at the plasma membrane, they are pumping so long as there are substrate and protons to pump. Therefore, more transporters at the PM equals more substrate uptake. A toxic uracil analog called 5-Fluorouracil (5-FU) can be used to indicate uptake of substrate and thus relative amount of transporters on the PM. 5-Fluorouracil gets incorporated in RNA and inhibits RNA processing, thus inhibiting protein production and cell growth (*59*). Cells with more Fur4 transporters on the plasma membrane will take up more 5-FU and will grow more slowly. Hence, sensitivity to 5-FU is an indicator of recycling of Fur4 to the plasma membrane – more uptake of the drug is indicative of more recycling. The *ubp2*Δ strain MCY66 is more sensitive to 5-FU than wildtype, consistent with increased recycling of Fur4 (Fig. 2.6).

As a third approach, we used western blotting to look at relative amounts of Fur4 degradation, plus or minus treatment. Looking at the amount of free GFP indicates the amount of Fur4-GFP protein that has entered and/or been degraded in the vacuole (since GFP is slow to degrade and can persist in the vacuole for hours). Under steady state conditions, more free GFP was present in the wildtype strain versus the *ubp2*Δ strain MCY66 (normalized to Snf7 loading control). This indicates more degradation of Fur4 in wildtype cells than in *ubp2*Δ cells. After heat shock, there is still more free GFP in wildtype than in *ubp2*Δ cells (Fig. 2.7). This result is consistent with microscopy and growth assay data, and supports the idea that *ubp2*Δ causes increased recycling of Fur4.

These results from microscopy, growth assay, and western blotting strongly suggest that Ubp2 has other functions in addition to DUB activity, or that there is further regulation at the early endosome in addition to signal mediated

governance by ubiquitin. If our EQRS model is accurate, and Ubp2 functions in retention of cargo with the aid of the other proteins, the complete deletion of Ubp2 likely disrupts the EQRS complex. This explains why deletion of Ubp2 increases recycling – because retention is impaired and even ubiquitinated proteins escape the early endosome. Thus, we wanted to look at how an active site mutant version of Ubp2 would affect Fur4 trafficking, versus a complete deletion of Ubp2 protein. This would allow us to look at the singular effect of loss of DUB activity, without disrupting retention activity.

2.3.4 Complementation with Ubp2 Active Site Mutant

We engineered a mutation (C745A) in the active site of Ubp2. This mutation abolishes the deubiquitinase activity of Ubp2, while still allowing it to bind ubiquitin and to interact with its normal protein partners (*56*, *60*). We replaced endogenous Ubp2 with *ubp2*^{C745A}, followed Fur4-GFP trafficking in the cell, and used growth assays and western blots to look at how the mutant affected degradation of Fur4. If Ubp2's role in QC at the early endosome is simply deubiquitination, a deletion and a point mutant should influence the system in the same way; they should lead to the same outcomes. However, if Ubp2 has a broader role in QC – retention of cargo proteins – then the two manipulations will yield very different results. *ubp2*Δ causes increased recycling, we presume, because of loss of retention of Fur4. We predicted *ubp2*^{C745A} would, on the contrary, cause increased degradation of Fur4, because retention would still be possible while deubiquitination would not.

Interestingly, when the mutant *ubp2*^{C745A} (pKF19) was added as

complement to *ubp2*Δ strain (MCY66), Fur4 trafficking as seen by microscopy was clearly different from the *ubp2*Δ without complement added, and wildtype (Fig. 2.5). Fur4 was then degraded more efficiently. In fact, even under steady state conditions with no treatment, Fur4 was already visible in higher amounts inside the cell (in endosomes/MVBs/vacuole) than in *ubp2*Δ alone or wildtype (Fig. 2.5). This phenotype suggests decreased recycling of Fur4.

5-Fluorouracil growth assays were consistent with microscopy results. A full deletion of UBP2 grew the worst of strains tested (Fig. 2.6), as would be expected with more recycling of Fur4 (more uptake of 5-FU). On the contrary, complementing the *ubp2*Δ cells with mutant $ubp2^{C745A}$ (pKF19) rescued their growth, suggesting increased Fur4 degradation. Surprisingly, wildtype strain SEY6210 and the wildtype UBP2 complement (pKF17) in *ubp2*Δ strain grew the best. We had predicted the strain with $ubp2^{C745A}$ complement would have grown best because of high amounts of Fur4 degradation.

Western blot showed overall lower levels of total Fur4 protein in $ubp2^{C745A}$ complement versus just *ubp2*Δ (Fig. 2.7). Also, with the mutant complement there wasn't much change in relative Fur4 degradation before or after heat shock, while in wildtype and *ubp2*Δ there is a clear increase in degradation following heat shock. Interestingly, the relative amount of Fur4 degradation occurring in $ubp2^{C745A}$ complement *before* heat shock, is about the same amount that occurs in *ubp2*Δ or wildtype *after* heat shock. The same trend is evident when looking at relative amount of Fur4 protein normalized to Snf7: the amount of Fur4 present in $ubp2^{C745A}$ complemented cells *before* heat shock is about the same amount of Fur4 present in *ubp2*Δ or wildtype *after* heat shock. So it appears even at steady

state there is probably more degradation occurring with the *ubp2*^{C745A} mutant than in *ubp2Δ* alone or wildtype cells. This differs slightly from the 5-FU growth assay, but is more like what was expected. As predicted, while *ubp2*Δ causes increased recycling of Fur4, *ubp2*^{C745A} complement reverses the phenotype causing increased degradation of Fur4. Thus far, results mostly support our EQRS model, or at least do not reject it.

2.3.5 Double Knockout of UBP2 and UBP7

As stated, there are two DUBs likely working at the early endosome. Deletion of UBP7 did not give such a strong phenotype, as compared to deletion of UBP2, which is why we focused on Ubp2. But if both DUBs were simultaneously deleted we predicted we might see more degradation, since this would theoretically nearly abolish deubiquitination at the early endosome. And microscopy and western blot data weakly suggest this may be correct.

Western blot quantification shows slightly increased degradation of Fur4 in the double knockout after heat shock, versus wildtype and single *ubp2*Δ (Fig. 2.7). Interestingly, when complemented with *ubp2*^{C745A}, the double knockout strain follows a similar trend as the single *ubp2*Δ complemented: degradation of Fur4 is increased even before heat shock, and there is little change in Fur4 degradation after heat shock.

By microscopy, there is not a clear phenotype of *ubp2*Δ-*ubp7*Δ at steady state. Upon treatment though, our *ubp2*Δ-*ubp7*Δ strains, KRY5-1 and KRY5-2, had more efficient downregulation and degradation than wildtype or *ubp7*Δ alone. After 30 minutes at high uracil concentration, the double knockout strains had

mostly endocytosed Fur4 off the plasma membrane, while a lot was remaining at the PM in wildtype cells at 30 minutes. The double knockout strains showed most of the GFP signal in vacuoles or endosomes/MVBs, while wildtype had less internal signal.

2.3.6 Deletion of EQRS Component Rup1

Rup1 is known to physically interact with and tether Ubp2 to Rsp5 (*50*). The purpose this serves is not well understood, but it has been shown that Rup1 modulates activity of Rsp5 and Ubp2 (*50*, *56*). Could Rup1 be involved in the recruitment of both enzymes to the early endosome? If so, we predict deletion of RUP1 would completely dismantle the system.

We looked by microscopy at Fur4-GFP trafficking in a *rup1*Δ strain (MCY64) (Fig. 2.8). A phenotype was not very conspicuous, but upon induced downregulation by uracil addition, we noticed quicker internalization (or just less recycling), and possibly, a delay in Fur4 reaching the vacuole. 60 minutes after uracil addition, many of these cells still showed a large amount of GFP signal coming from endosomes/MVBs, not as much from the vacuole (where most signal is in wildtype after 60 minutes uracil). It seems Fur4 is still efficiently endocytosed in *rup1*Δ cells, and possibly, undergoes less recycling than wildtype. Also, Fur4 becomes stuck in endosomes and doesn't make it to the vacuole as efficiently as usual in *rup1*Δ cells.

By growth assay, *rup1Δ* appears less sensitive to 5-FU than wildtype. This data supports the idea of a slight recycling defect when Rup1 is absent. If this is the case, deletion of RUP1 does not fully dismantle the system as

expected. However, it may perturb both recycling and degradation, which could explain why we see Fur4 stuck in endosomes/MVBs.

2.3.7 Deletion of EQRS Component Hua1

By microscopy, deletion of HUA1 alone had no real discernible phenotype. However, in a 5-FU growth assay *hua1*Δ did appear to have a growth advantage over wildtype. It is possible that deletion of HUA1 slightly decreases recycling or increases degradation, but the effect is so mild it is not detectable by microscopy.

2.3.8 Double Knockout of HUA1 and RUP1

The lack of obvious phenotypes from deletion of HUA1 or RUP1 may be because the EQRS system has some redundancy. We thought combining HUA1 and RUP1 deletions in one strain might reveal a clearer phenotype.

A phenotype did become more evident in RUP1-HUA1 double knockout cells after overnight growth. Typically after overnight growth, when cells have entered stationary phase and gone into starvation mode, almost all Fur4 has been endocytosed and is in the vacuole being degraded. This is what was observed in wildtype cells and in *ubp2*Δ-*ubp7*Δ cells: almost all GFP signal was seen in the vacuole. *rup1*Δ-*hua1*Δ cells, however, were loaded with endosomes containing Fur4-GFP and even had some signal remaining at the plasma membrane after overnight growth to stationary phase. This supports the idea that deletion of RUP1 causes a delay in cargo reaching the vacuole, while simultaneous deletion of HUA1 seems to amplify the effect. The strain has not yet been analyzed by western blot or growth assay.

2.4 Discussion

We confirmed that deletion of UBP2 yields increased recycling, but that complementation with a Ubp2 active site mutant increases degradation. Thus, a simple concept of tug-of-war between Rsp5 ubiquitinating and Ubp2 deubiquitinating cargo proteins at the early endosome is insufficient to account for the decision to degrade or recycle, respectively. Additionally, there are other endosome localized proteins that interact with Rsp5 and Ubp2 that cannot be ignored, for their interaction must serve a purpose. All four proteins – Rup1, Hua1, Rsp5, and Ubp2 – are ubiquitin binding proteins, which suggests a mode for engagement with cargo. We also know Rup1 has a modulatory effect on Rsp5 and Ubp2, based on previously published data (*50*, *56*). It has been suggested that deletion of Ubp2 results in increased recycling of cargo because of autoubiquitination of Rsp5 without competing deubiquitination, thus resulting in decreased ubiquitin ligase activity (*56*). But our Ubp2 active site mutant data rejects that idea, as the mutant should also cause increased Fur4 recycling, if loss of DUB activity and thus lower amounts of Rsp5 were the explanation.

Our EQRS model is bolstered by the UBP2 data. Complete deletion of UBP2 would disrupt the EQRS complex and even ubiquitinated cargo could escape retention. This accounts for the increased recycling observed in cells with *ubp2*Δ. However, with an active site mutant version of Ubp2, where DUB activity is diminished, the EQRS complex can still form and retain cargo. This accounts for increased degradation seen in *ubp2*Δ cells complemented with $ubp2^{C745A}$.

Furthermore, Hua1 may have a scaffolding role or be involved in handoff

to ESCRT-0, as it is known to physically interact with Hse1 (*25*). Rup1 could also have a hand in maturation of endosomes. These are just ideas that require further testing, but it could explain why *rup1*Δ and *rup1*Δ-*hua1*Δ cells have cargo stuck in endosomes with delayed progression to vacuoles. When the cells should thoroughly downregulate (i.e., during starvation), Fur4 may be endocytosed normally, but become stuck at endosomes during the ubiquitination-deubiquitination cycle, yet with some escaping back to the plasma membrane (but almost immediately being endocytosed again).

It appears Ubp7 does not play a crucial role at the endosomes as does Ubp2. UBP7 deletion alone does not have a strong phenotype. However, combination with UBP2 deletion does appear to increase degradation, so it may be that deletion of the endosomal DUBs drastically reduces deubiquitination and ability to recycle.

The microscopy and growth assay data seems reliable since these experiments have been repeated and given the same results. However, it must be noted that the western blot data comes mostly from one blot. A repeat blot showed some of the same trends (but weak) or else no significant trends. So, more blots really need to be performed to get significant data. Also, in our sample preparation procedure, unfortunately, there tends to be degradation occurring during the prep. We have since found a new protocol that should quickly stop all activity in the cell to prevent degradation occurring during the preps. Western blots need to be repeated with this new sample prep procedure, and for the purpose of providing replicate data.

Table 2.1: Strains and Plasmids Used in This Study

Strains	Description	Genotype	Source
SEY 6210	WT	MATα *leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	(*61*)
MYY808	*rsp5-1*	MYY808 MATα, MDM1, *smm1, his3, leu2, ura3*	(*62*)
MCY64	*rup1*Δ	SEY 6210, *rup1*::*KanMX*	This study
MCY65	*ubp7*Δ	SEY 6210, *ubp7::KanMX*	This study
MCY66	*ubp2*Δ	SEY 6210, *ubp2::KanMX*	This study
KRY1	Rup1-GFP	SEY 6210, *Rup1-GFP::KanMX*	This study
KRY2	Ubp2-GFP	SEY 6210, *Ubp2-GFP::KanMX*	This study
KRY4	*hua1*Δ	SEY 6210, *hua1::KanMX*	This study
KRY5-1	*ubp2*Δ*ubp7*Δ	SEY 6210, *ubp2::TRP1, ubp7::KanMX*	This study
KRY8	*hua1*Δ*rup1*Δ	SEY 6210, *hua1::TRP1, rup1::KanMX*	This study
Plasmids			
pJK19	P(CUP1)-FUR4-GFP	URA3 (pRS416) P(CUP1)-FUR4-GFP	(*35*)
pKF17	P(UBP2)-UBP2	LEU2 (pRS415) P(UBP2)-UBP2	This study
pKF19	P(UBP2)-*ubp2*^{C745A}	LEU2 (pRS415) P(UBP2)-*ubp2*^{C745A}	This study
pMB103	P(VPS4)-*vps4*^{E233Q}	URA3(pRS416) P(VPS4)-*vps4*^{E233Q}	(*63*) Created by M. Babst
p415Cherry DID2	P(VPS21)-mCherry-DID2	LEU2(pRS415) P(VPS21)-mCherry-DID2	(*64*)

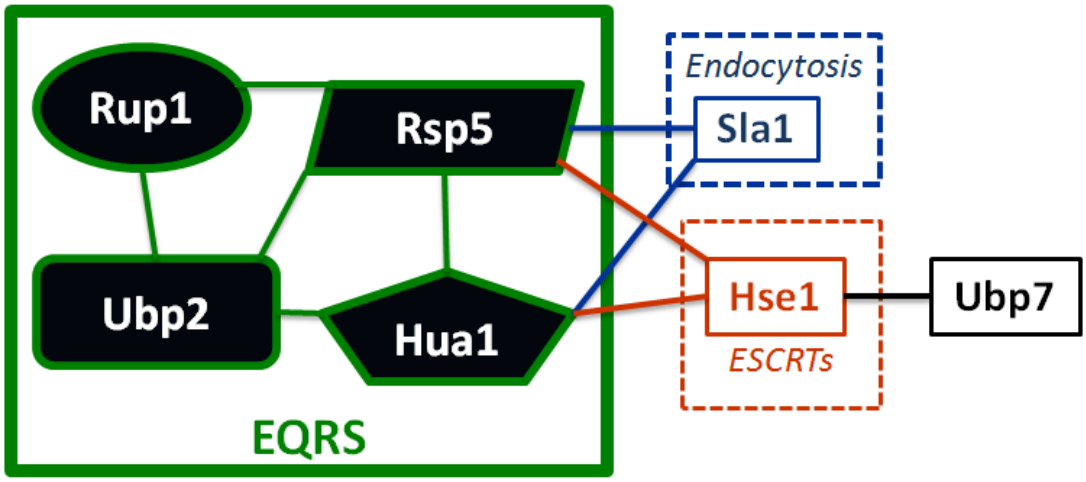


Fig. 2.1: Protein Interaction Map
Connecting lines show physical interactions between proteins.

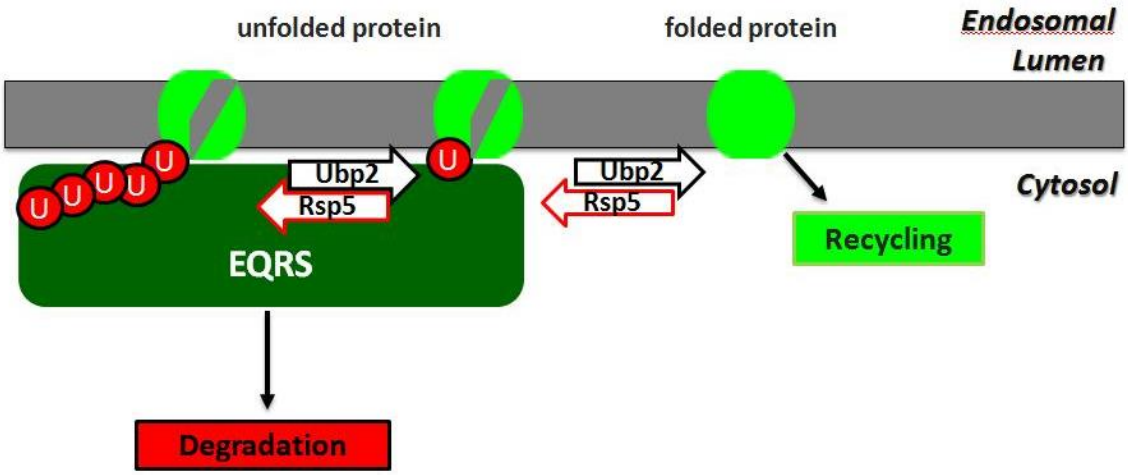


Fig. 2.2: EQRS (Endosomal Quality control & Retention System) Model
In this model, the green EQRS box represents a complex of the four key proteins – Ubp2, Rsp5, Rup1, and Hua1. A protein (e.g., Fur4) cycles through dynamic deubiquitination and (poly)ubiquitination. Whatever remains ubiquitinated can be caught by the retention complex and held back in the endosome. Without ubiquitin moieties attached, EQRS is not able to retain Fur4 and it escapes, recycling to the PM. Anything held back by EQRS goes along with the maturing endosome to be degraded in the vacuole/lysosome. The EQRS machinery likely is reused by falling off around the late endosome stage as the cargo is handed over to the ESCRTs.

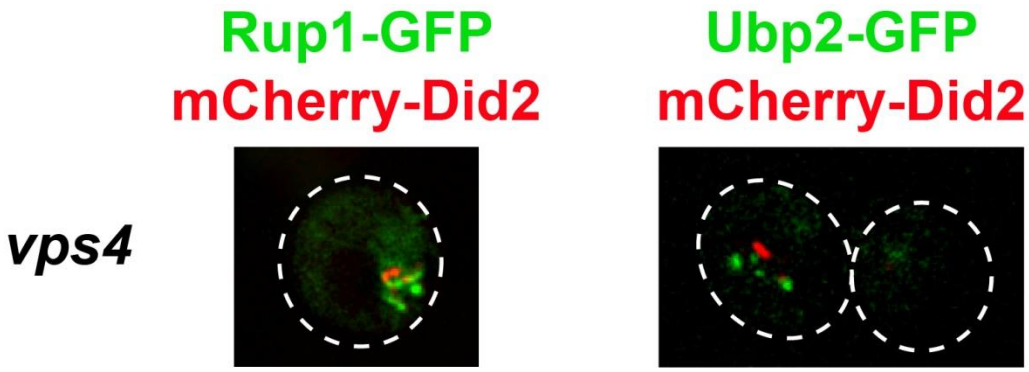
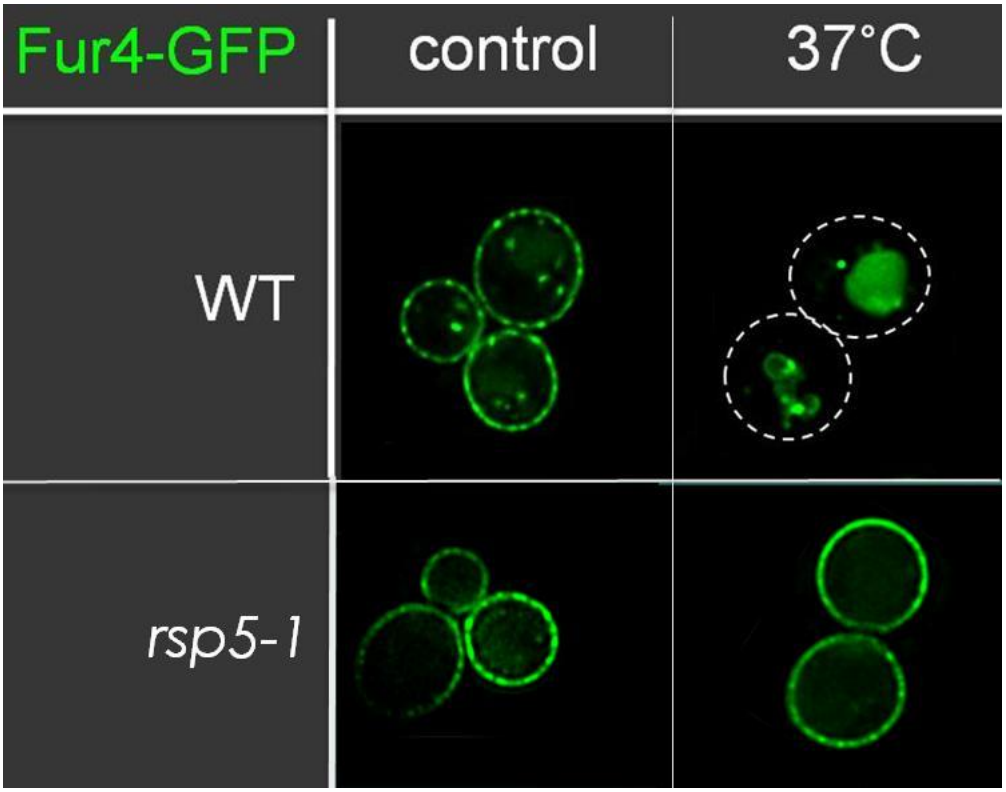


Fig. 2.3: Localization of EQRS Proteins
The plasma membrane of the cell has been outlined in white dashed line. These cells express a plasmid containing a *vps4* dominant negative mutant which causes accumulation of late endosomes. Rup1 or Ubp2 (as indicated) is present where GFP is seen. The ESCRT factor Did2 is present where red mCherry is visible, and is used as a marker of late endosomes. The images show no overlap of GFP and mCherry, revealing that EQRS components accumulate on compartments that are distinct from late endosomes/MVBs. This suggests EQRS functions upstream of the ESCRTs. Additionally, without the vps4 mutation, Rup1 and Ubp2 are more diffuse in the cell, and harder to specifically localize. The fact that they accumulated on late endosomes here suggests that ESCRT factor vps4 may be necessary for recycling of the EQRS components.

a.



b.

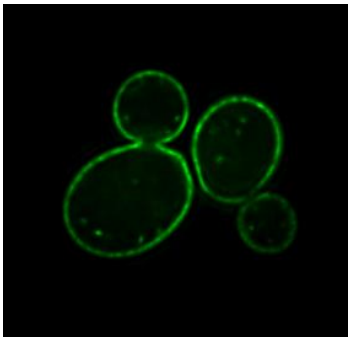


Fig. 2.4: Microscopy on *rsp5-1* Cells
a. Wildtype shows very efficient downregulation of Fur4 after 60 minutes heat shock at 37°C, while Fur4 is stabilized on the PM in *rsp5-1* strain before and after heat shock.
b. Pictured is Fur4-GFP in *rsp5-1* after 60 minutes +uracil. The signal in dots close to the plasma membrane indicates endosomal compartments. Efficiency of endocytosis has been greatly reduced in *rsp5-1*, but clearly not obliterated.

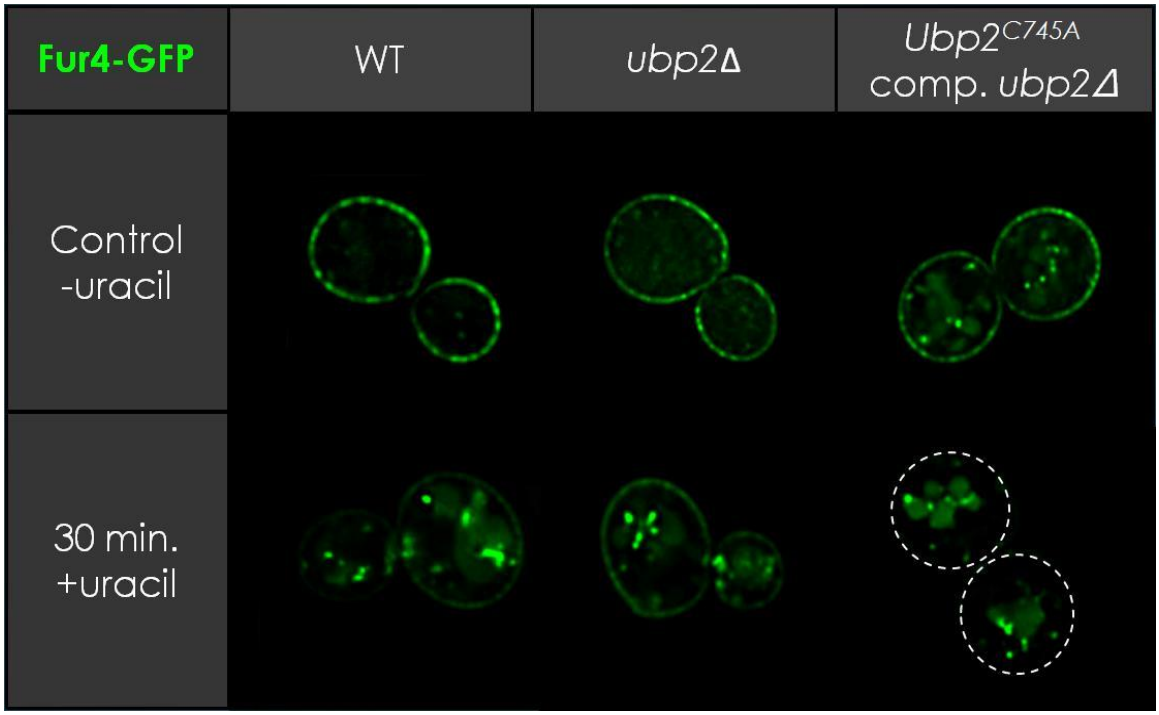


Fig. 2.5: Microscopy on *ubp2*Δ Cells
White dashed lines mark the PM. WT shows normal downregulation after uracil addition. Most GFP signal is in the vacuole, with a little in MVBs. *ubp2*Δ shows much less GFP in vacuoles than WT, and much more still remaining at the PM, indicative of increased recycling of Fur4. But when complemented with Ubp2 active site mutant, there is a switch to increased degradation. It looks very similar to WT after uracil, with no signal remaining at the PM. Before uracil, the complement shows much more internal signal, so there is increased degradation even at steady state.

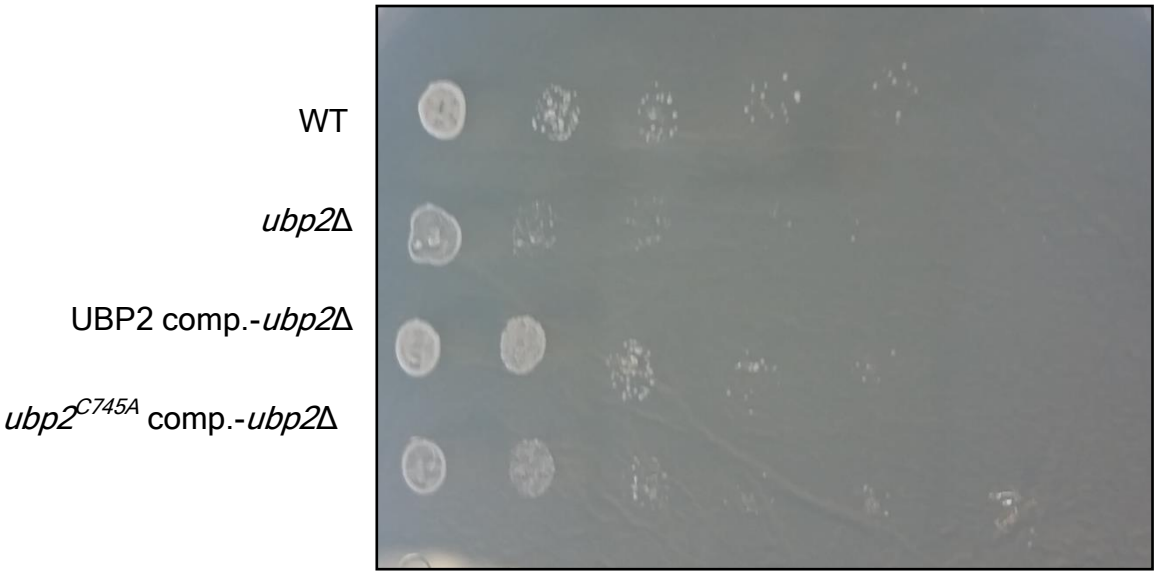


Fig. 2.6: 5-FU Growth Assay with *ubp2*Δ Cells
The UBP2 complement and WT grew best, which indicates less Fur4 on the PM (more degradation). *upb2*Δ was most sensitive to 5-FU, indicating more Fur4 on the PM (more recycling).

a.

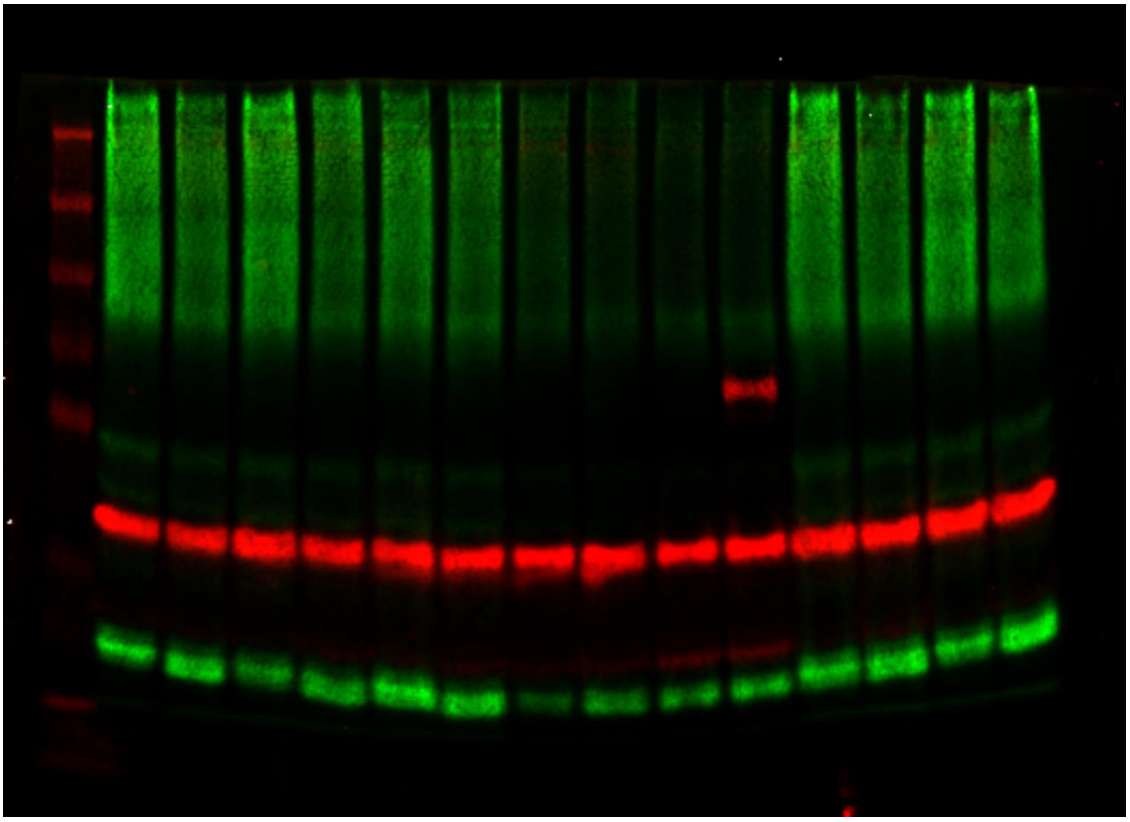
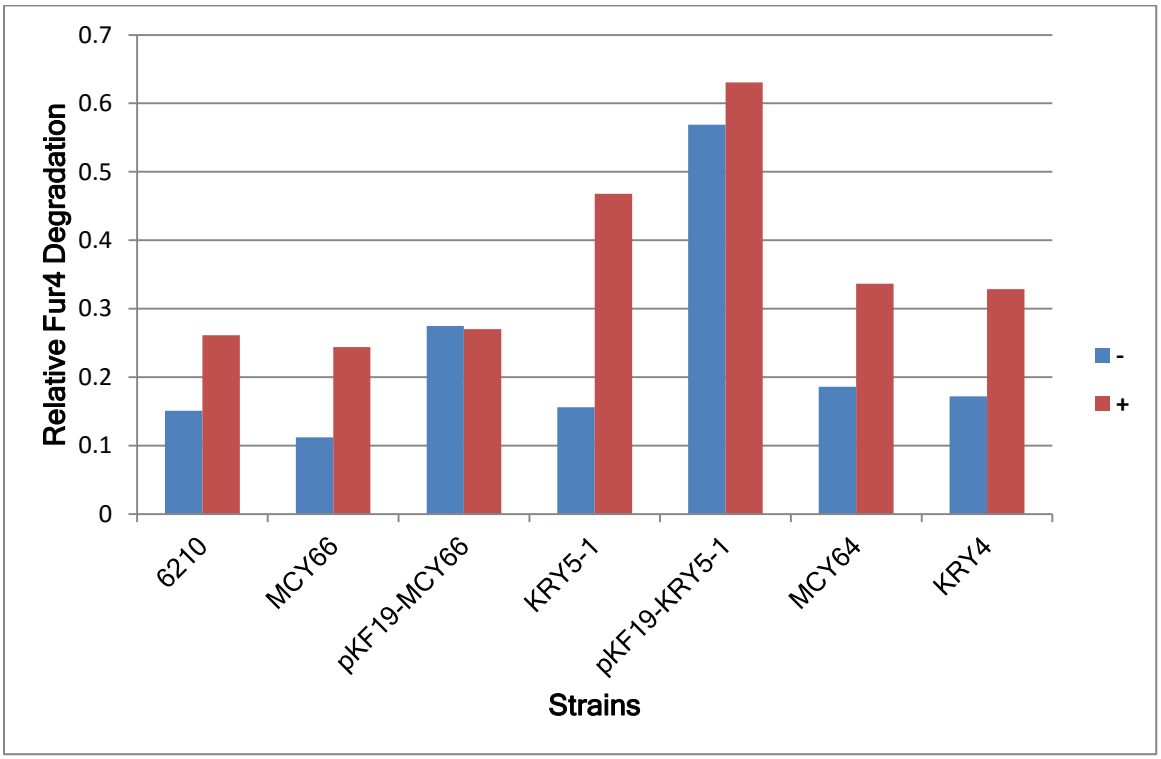
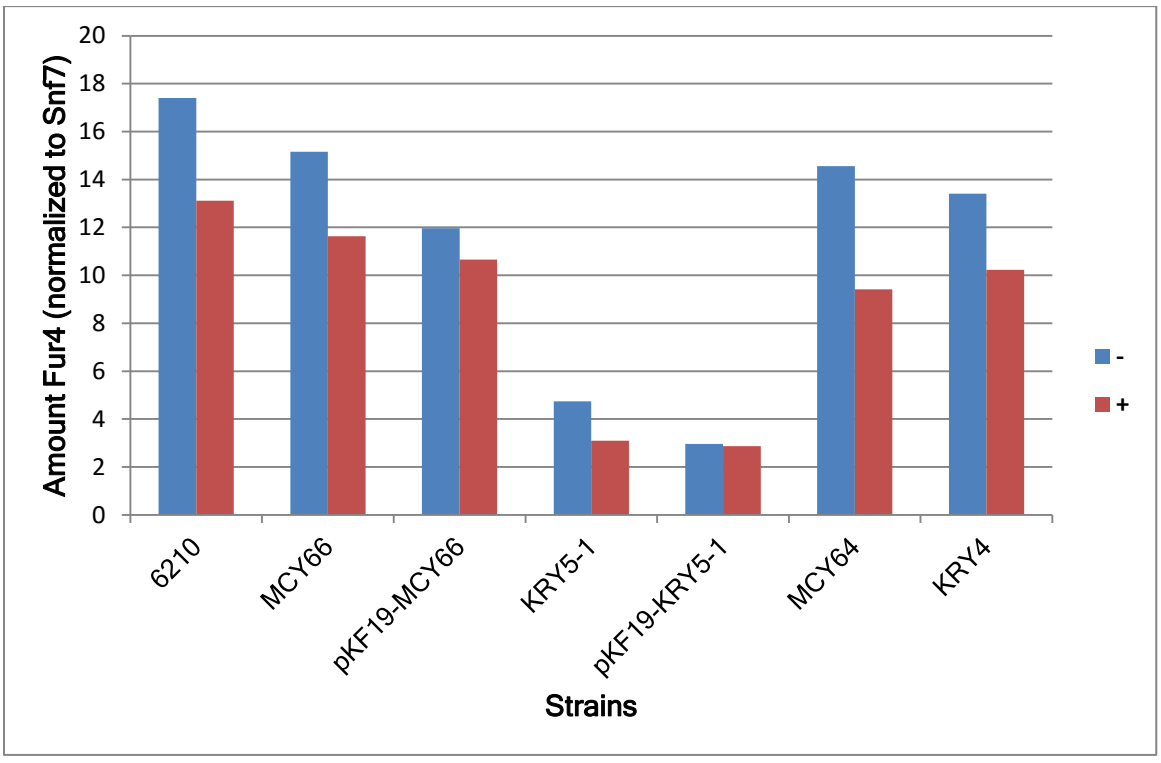


Fig. 2.7: Western Blot Data
a. Blot Image: Lanes match in order with the quantification graphs on the following page. The overall trend visible here is a decrease in Fur4 protein after heat shock (every 2nd lane) and lower Fur4 protein amounts overall in the double *ubp2*Δ-*ubp7*Δ (lanes 7-10).
b. Quantification: -/+ equals before (-) or after (+) heat shock treatment for 20 minutes.

b.


Relative Fur4 Degradation
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
6210
MCY66
pKF19-MCY66
KRY5-1
pKF19-KRY5-1
MCY64
KRY4
Strains
-
+


Amount Fur4 (normalized to Snf7)
20
18
16
14
12
10
8
6
4
2
0
6210
MCY66
pKF19-MCY66
KRY5-1
pKF19-KRY5-1
MCY64
KRY4
Strains
-
+

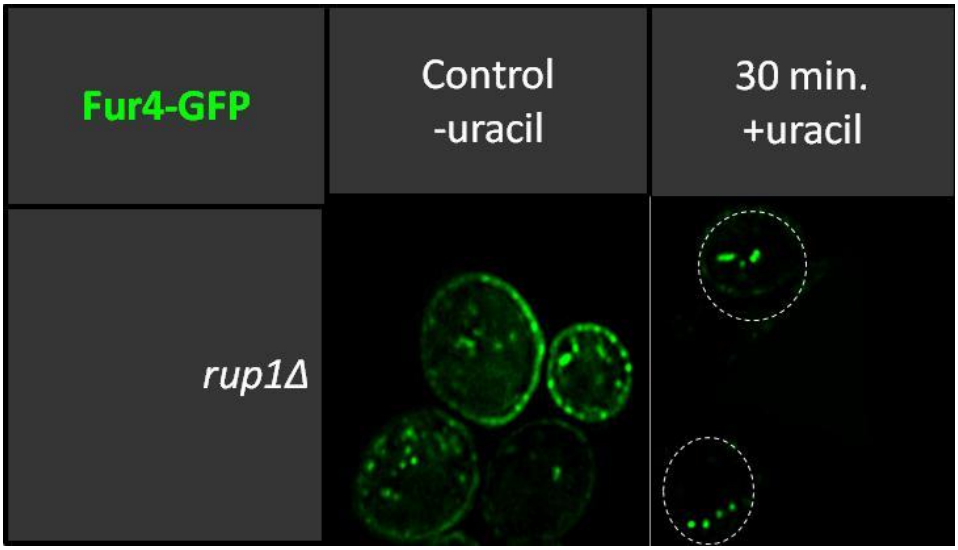


Fig. 2.8: Microscopy on *rup1*Δ Cells
After 30 minutes +uracil, all GFP signal appears in endosomes/MVB, instead of the majority being in the vacuole as with WT (refer to Fig. 2.4).

CHAPTER 3

CONCLUSION

3.1 Concluding Remarks and Future Directions

Maintaining functional proteins at the plasma membrane is vital for nutrient uptake, ion exchange for maintenance of membrane potential, and for cell-to-cell communication. Proteins can become unfolded or unstable on the plasma membrane, so a quality control system must be in place to take care of these proteins. Transporters like Fur4 may become unstable just as a byproduct of their natural pumping mechanism. Hence, Fur4 requires monitoring and removal from the plasma membrane to preserve cell integrity. At the same time, cells need to balance integrity with energy conservation, so there is a second QC system in place at the endosome to determine whether a PM protein really needs to be degraded or if the protein is still able to function and should be conserved. If a protein appears unfolded, it is quickly endocytosed to prevent it from being detrimental to the cell, possibly causing loss of membrane potential among other problems. After endocytosis, the protein can either continue down the MVB pathway to be degraded in the lysosome/vacuole, or it can be recycled back to the plasma membrane. The choice between these two fates is made at the early endosome. How this decision is made is incompletely understood. A widely

accepted idea in the field is that ubiquitination status determines a protein's fate, and that the processes of ubiquitination and deubiquitination occurring at the early endosome control that fate. However, numerous experiments on *ubp2*Δ strains suggest a more complex system of quality control at the early endosome. It's clear that deletion of UBP2 causes increased recycling of proteins to the plasma membrane, the polar opposite phenotype expected. So how can this be explained?

That is how the EQRS model was born, to explain these unexpected results. There are many open questions and theories that should be explored through further research of this topic. A future goal is to understand how the retention complex is organized and its timing. What are the functional roles of all the key players in our EQRS model? We still do not know what Hua1 does. Which components are essential? Ubp7 likely is not essential since its deletion did not have an obvious phenotype, and there is probably some redundancy with Ubp2. How are the EQRS factors recruited to the endosome? At what point does EQRS hand off cargo to the ESCRTs? We believe EQRS functions upstream of the ESCRTs, so there is likely a step where EQRS leaves prior to or in conjunction with ESCRT recruitment. Answering these questions will help us understand the vital second quality control system at work at the early endosome.

In spite of leaving us with many questions, the present work has added support to our EQRS model for the most part, leaving us with many future directions in which to go.

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